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Expression of Insulin-Like Factor 3 Protein in the Rat Testis during Fetal and Postnatal Development and in Relation to Cryptorchidism Induced by *in Utero* Exposure to Di (*n*-Butyl) Phthalate

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Cryptorchidism is a common reproductive abnormality, possibly resulting from abnormal hormone production/action by the fetal testis. Insulin-like factor 3 (Insl3) is thought to be involved in gubernaculum development and transabdominal testicular descent, but its importance is unclear, due partly to lack of suitable Insl3 antibodies. We generated (by genetic immunization) and validated a novel antirat Insl3 antibody, which we used to characterize immunoexpression of Insl3 in rat Leydig cells (LCs) from fetal life until adulthood and its relationship to cryptorchidism. Immunoexpression was strong on embryonic day (E) 17.5 and E19.5 and from 35 d of age onward but weak from E21.5 until puberty. Because *in utero* exposure to di (*n*-butyl) phthalate (DBP) induces cryptorchidism and suppresses Insl3 gene expression, we investigated Insl3 protein expression in fetal and adult rats exposed to 500 mg/kg·d DBP from E13.5 to E21.5. Expression on E17.5 and E19.5 decreased dramatically after DBP exposure, but there was no consistent correlation between this suppression and abnormal testis position. We also compared expression of Insl3 and P450 side-chain cleavage enzyme in fetal testes from rats exposed *in utero* to DBP or flutamide (50 mg/kg·d). DBP treatment suppressed expression of both P450 side-chain cleavage enzyme and Insl3 at E19.5, but flutamide exposure had no effect on either protein, demonstrating that Insl3 expression in fetal rat LCs is not androgen regulated. In adult rats, Insl3 expression was suppressed in 80% of cryptorchid and 50% of scrotal testes from rats exposed to DBP, suggesting that prenatal DBP exposure also leads to maldevelopment/malfunction of the adult LC population in some animals. (*Endocrinology* 146: 4536–4544, 2005)

FAILURE OF TESTICULAR descent (cryptorchidism) is the most common congenital abnormality found in children, occurring in at least 2–4% of male births (1–3). Although the causes of cryptorchidism are probably multifactorial, it is thought that deficiencies in production or action of testosterone and/or insulin-like factor 3 (Insl3), also known as relaxin-like factor (RLF), by the fetal testis are likely to be important. Furthermore, cryptorchidism is often associated with other abnormalities of the male reproductive system, such as hypospadias, low sperm counts, and testicular germ cell cancer, associations that have led to the hypothesis of a testicular dysgenesis syndrome (4). Lifestyle/environmental factors are thought to be involved in the etiology of testicular dysgenesis syndrome (4, 5).

It is generally accepted that the role of testosterone in normal testicular descent centers on the final, inguinoscrotal phase (6, 7), and it is disorders of this phase of descent that account for most cases of cryptorchidism, especially those that resolve spontaneously in the first few months after birth (6). More recently, a role for Insl3 in development of the gubernaculum (8, 9) and in the transabdominal phase of testicular descent, has emerged (for reviews see Refs. 10–12) after demonstration of impaired gubernacular development and testis descent in Insl3$^{-/-}$ mice (13, 14). The latter findings prompted investigations of the potential importance of mutations/polymorphisms in the Insl3 gene in cases of human cryptorchidism, but so far these have not provided evidence of a major role for Insl3 gene defects in testicular maldescent (15–19). Therefore, although the Insl3 knockout studies in mice are highly suggestive of a key role for this hormone in testis descent, definitive evidence for an important role in spontaneous cases of cryptorchidism is still lacking. One obstacle to such studies has been the lack of availability of good antibodies to Insl3 that would enable its detection in tissue sections and/or its measurement in blood (11).

An Insl3 antibody that works in mice has been available (20), and just recently antibodies that allow detection of Insl3 in blood in humans have become available (21, 22), but otherwise there have been major problems in generating useable

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Abbreviations: DBP, Di (*n*-butyl) phthalate; E, embryonic day; EDS, ethane dimethane sulfonate; Insl3, insulin-like factor 3; LC, Leydig cell; P450scc, side-chain cleavage enzyme; TBS, Tris-buffered saline; TBST, TBS containing Tween 20.

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antibodies to Insl3, probably because of its complex structure (11). As a result, there are few definitive data on age-related changes in Insl3 expression/production by the Leydig cells (LCs) and whether changes in Insl3 protein expression correlates in any way with failure of testicular descent. To address these deficiencies, we developed an antibody to rat Insl3, using genetic immunization and, after validation, have used this antibody to characterize Insl3 protein expression in LCs in the rat testis throughout fetal and postnatal life. We also investigated Insl3 immunoexpression in the fetal testis in a model system involving *in utero* exposure of rats to di (*n*-butyl) phthalate (DBP), in which a high incidence of testicular maldescent is induced in association with alterations in gubernacular development and suppression of Insl3 gene expression (23). Our results demonstrate major changes according to both fetal and postnatal age in Insl3 expression in the LCs but do not show a consistent relationship in fetal life between testicular malposition and reduced Insl3 expression in the fetal LCs, although such a relationship is evident in the adult cryptorchid testis.

Materials and Methods

Animals and treatments

Wistar rats were maintained in our own animal facility according to United Kingdom Home Office guidelines and were fed a soy-free breeding diet (SDS, Dundee, Scotland). For some experiments, time-mated females were treated from embryonic day (E) 13.5 to E21.5 with either 0 (control) or 500 mg/kg DBP (Sigma-Aldrich Co. Ltd., Dorset, UK) in 1 ml/kg corn oil administered daily by oral gavage. This dose has been shown previously to result in a high incidence (~75%) of unilateral and/or bilateral cryptorchidism in Wistar rats as well as other dysgenetic changes in the testis (24, 25). Other time-mated females were treated from E15.5 to E21.5 with either 0 (control) or 50 mg/kg flutamide (Sigma-Aldrich) in 1 ml/kg corn oil administered daily by oral gavage. The DBP and flutamide used were both 99% pure according to the supplier's data.

To induce total ablation of the LCs in the adult rat testis (26, 27), males aged approximately 75 d were injected ip with 75 mg/kg ethane dimethane sulfonate (EDS) in 1:3 (vol/vol) dimethylsulfoxide-water and were then killed 6 d later and their testes perfusion-fixed with Bouins solution, as described previously (28, 29). In one instance, testes from control and EDS-treated rats were not fixed but were cut into pieces and rapidly frozen on dry ice and then stored at −80 C before protein extraction for Western blotting.

For RNA studies time-mated, untreated pregnant female Sprague Dawley rats were purchased from Elevage Janvier (Le Genest Saint Isle, Laval, France).

Sample collection and processing

Fetal samples. Control and DBP-treated pregnant dams were killed by inhalation of carbon dioxide on E15.5 (n = 2 control), E17.5 (n = 2 control, n = 3 DBP), E19.5 (n = 3 control, 3 DBP), E20.5 (n = 1 control, 2 DBP), or E21.5 (n = 2 control, 2 DBP). Fetuses were removed, decapitated, and placed in ice-cold PBS (Sigma-Aldrich). After incision of the abdominal wall, the location of both testes was carefully recorded as being high abdominal (adjacent to the kidney), midabdominal, or inguinal (normally descended). Testes were then removed via microdissection and either fixed for 1 h in Bouins solution or transferred to an Eppendorf tube and frozen immediately on dry ice for subsequent protein extraction. Fixed testes were transferred to 70% ethanol and then weighed before being processed for 17.5 h in an automated Leica TP1050 processor and embedded in paraffin wax. Gubernacula were also microdissected from some fetuses at E19.5, fixed, and embedded as described above for the testes. Representative fetuses from the aforementioned litters were subsequently used for the immunohistochemical and protein expression studies detailed below.

For RNA studies, dams were anesthetized by an ip injection of 40 mg/kg sodium pentobarbital (Sanofi-Synthélabo, Libourne, France) on E18.5, E19.5, and E20.5. Gubernacula were removed from male fetuses by microdissection and frozen immediately in liquid nitrogen.

Postnatal samples. Male rats aged 4, 10, 15, 25, 35 or 90 d (adults) were anesthetized via flurothane inhalation and then killed by cervical dislocation. Testes were carefully inspected for normality of the epididymis and vas deferens and then removed, weighed, fixed for 5–6 h in Bouins solution, and then transferred into 70% ethanol. Adult testes were halved after approximately 3 h fixation to aid penetration of the fixative. Testes were embedded in paraffin as described above. The results reported in the present studies derive from males from at least two to three separate litters per age group. At autopsy, testicular position was classified as high abdominal (at level of the kidney), midabdominal, inguinal, or scrotal, which enabled classification of testes in 90-d males into cryptorchid or scrotal groups. In controls, all testes were scrotal in position. The prevalence of unilateral and bilateral cryptorchidism in DBP-exposed adult males was 75 and 25% respectively.

For RNA studies, male rats aged 4 or 90 d (adults) were killed as described above. Gubernacula were microdissected from 4-d animals whereas testes, efferent ducts, and kidneys were all obtained from adult rats and all organs frozen immediately in liquid nitrogen. The testes from some adult animals were not frozen but were used instead for purification of LCs using a method previously described (30).

Extraction of testis protein

Whole testes from E17.5 fetal rats or small slices of testes from adult rats were used for extraction of protein. Frozen tissue was transferred to 1.5 ml Eppendorf tubes on ice, to which was added 75 (for fetal samples) or 200 μl (for adults) of ice-cold lysis buffer comprising 0.15 M NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1% (wt/vol) sodium deoxycholate, and 0.1% (wt/vol) sodium dodecyl sulfate and containing one tablet of Complete protease inhibitor (Roche Diagnostics GmbH, Mannheim, Germany) per 10 ml. Tissue was homogenized and kept on ice for 60 min. The homogenate was then centrifuged at 10,000 rpm for 5 min at 4 C and the supernatant removed. Before storage at −80 C, a small aliquot of supernatant was removed and protein concentration measured using the Protein Dc assay kit (Bio-Rad Laboratories, Hemel Hempstead, UK) according to the manufacturer's instructions. All samples were assayed in triplicate.

Generation of antirat Insl3 antibody

To generate a polyclonal antirat Insl3 antiserum, we used the genetic immunization approach, in which an expression vector is introduced into cells of the skin by ballistic DNA transfection, directing them to produce the desired antigenic protein and to present it to the animal's immune system, most likely in its native conformation (31). To construct an expression vector for the rat Insl3 protein, total RNA from adult rat testis was reverse transcribed using SuperScript II (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and subjected to PCR using ProofStart polymerase (QIAGEN, Hilden, Germany) and Insl3-specific fusiogenic primers. These were designed to amplify the complete coding sequence (32) consisting of leader peptide, B, C, and A chains and contained overhanging restriction sites for *Eco*RI and *Sal*I to allow directed ligation of the amplicon into the respective sites of the commercially available expression vector pDisplay (Invitrogen). To the 3′ end of the Insl3 sequence, a further coding sequence for the peptide stretch GGGDPREQ was added to serve as a spacer toward the myc epitope tag and membrane anchor encoded in the pDisplay vector, with the purpose of improving independent native folding of the Insl3 part within the resulting fusion protein.

The PCR product had the expected size, was restriction digested, gel purified, and cloned into pDisplay. Sequencing analysis revealed that no mutation in the Insl3 sequence had occurred during amplification and cloning. Expression capability of the construct was confirmed by transfection into COS7 cells using Lipofectamine 2000 (Invitrogen) followed by immunofluorescence staining of transfected and mock-transfected cells. The primary antibody was a monoclonal against the myc tag (EQKLISEED) of the fusion protein, using undiluted culture supernatant of the respective hybridoma line 9E10 (a gift from Nicholas Hunt, Evotec

AG, Hamburg, Germany), and the secondary antibody was Cy3 conjugated (AffiniPure goat antimouse IgG; Jackson ImmunoResearch Laboratories, Dianova, Hamburg, Germany). Only the transfected cells exhibited strong fluorescence (not shown), predominantly at the cell membranes in which the fusion protein is attached via a platelet-derived growth factor receptor transmembrane domain. This cell-specific staining was confirmed using a polyclonal rat antihuman Insl3 antiserum (22), apparently due to a moderate cross-reactivity of the antihuman antibody with rat Insl3 (data not shown).

Two rabbits were immunized with the expression construct using a Helios gene gun (Bio-Rad, Munich, Germany) essentially as described previously (31), with the following modifications: 12 shots were performed per animal at each immunization, and 10 d before killing, one rabbit was injected with 200 μl of a membrane preparation in PBS from one T75 flask of COS7 cells 40 h after transfection with the Insl3 expression construct. Immediately before harvesting, these cells were preincubated with the last bleed (1:500 in PBS, 30 min room temperature) from the second rabbit. Membrane preparation was performed as described previously (33). Immunofluorescence staining of transfected COS7 cells with the final antiserum (1:750) gave strong positive signal, whereas the preimmune serum, secondary antibody only, and staining of mock-transfected cells were all negative (not shown).

Immunohistochemistry

The antirat Insl3 antibody described above was used at a dilution of 1:100. P450 side chain cleavage enzyme (P450scc) and Insl3 receptor (LGR8), respectively, were localized using a rabbit polyclonal antibody (Chemicon, Chandlers Ford, UK) at a dilution of 1:400 and a goat polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a dilution of 1:40. Sections of 5 μm were mounted onto coated slides (VWR, Poole, UK), dewaxed, and rehydrated. Slides were incubated for 30 min in 3% (vol/vol) hydrogen peroxide in methanol to block endogenous peroxidase activity and then washed in Tris-buffered saline [TBS; 0.05 M Tris, 0.85% (wt/vol) NaCl (pH 7.4)]. Nonspecific binding sites were blocked with an appropriate normal serum diluted 1:5 in TBS containing 5% (wt/vol) BSA before the addition of the primary antibody and overnight incubation at 4 C. After washing in TBS, slides were incubated for 30 min with biotinylated swine antirabbit IgG secondary antibody (Dako, Ely, UK) diluted 1:500 in the blocking mixture. This was followed by 30 min incubation with horseradish peroxidase-labeled avidin-biotin complex (Dako). Immunostaining was developed by application of diaminobenzidine (liquid DAB$^+$; Dako), and slides were counterstained with hematoxylin, dehydrated, and mounted using Pertex mounting medium (Cell Path, Hemel Hempstead, UK).

Image capture

Nonfluorescent images were examined and photographed using a Provis microscope (Olympus Optical, London, UK) fitted with a Kodak DCS330 digital camera (Eastman Kodak, Rochester, NY). Fluorescent images were captured using a Zeiss LSM 510 Axiovert 100M confocal microscope (Carl Zeiss Ltd., Welwyn Garden City, UK). Images were compiled using Photoshop 7.0 (Adobe Systems Inc., Mountain View, CA).

Western blotting

Testis protein extract was boiled for 5 min in buffer comprising 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% (wt/vol) sodium dodecyl sulfate (all Sigma-Aldrich), 10% (vol/vol) glycerol (VWR) and 0.002% (wt/vol) Bromophenol Blue (Bio-Rad) before loading 10 μg of protein per lane onto a 12% (vol/vol) polyacrylamide gel. Samples were electrophoresed on a Mini-Protean II system (Bio-Rad) at 200 V for 45 min, and gels were then transferred to blotting buffer comprising 12 mM Tris base, 96 mM glycine (both Sigma-Aldrich), and 20% (vol/vol) methanol (VWR) and blotted onto a polyvinyl difluoride membrane (Immobilon-P; Millipore, Watford, UK) using a TE 22 transfer unit (Amersham Pharmacia Biotech, Little Chalfont, UK) run at 45 V for 4 h. Membranes were blocked for 1 h at room temperature in TBS containing 0.1% (vol/vol) Tween 20 (Sigma) (TBST) and 5% (wt/vol) skimmed milk powder and then incubated overnight at 4 C with Insl3 antibody diluted 1:1000 in the blocking mixture. The membranes were washed extensively in TBST and then incubated for 1 h at room temperature with peroxidase-conjugated goat antirabbit IgG diluted 1:4000 in TBST. After further washing in TBST, bound antibodies were detected using an ECLplus system and Hyperfilm exposure (both Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's instructions.

RNA extraction and RT-PCR analysis

Total RNA was extracted from each gubernaculum using a method previously described (34). Sequences encoding Insl3 and actin mRNA were amplified by RT-PCR. The Insl3 sequence-specific primers used were: sense 5′-CGCCAAGCTCTGTGGTCA-3′ and antisense 5′-CTGAGAAGCCTGGTGAGGAA-3′ (NCBI accession no. NM_053680). cDNA was prepared from 4 μg RNA using 40 ng of random hexanucleotides (Roche Molecular Biochemicals, Mannheim, Germany) and 200 U Moloney murine leukemia virus-reverse transcriptase (Promega, Madison, WI), according to the manufacturer's instructions. PCR was carried out on 40 ng cDNA in a final volume of 25 μl containing 0.6 U *Taq* polymerase (QIAGEN, Courtaboeuf, France) and 0.2 μM of each primer. Actin amplification was performed as a control for RNA quality, quantity estimation, and reverse transcriptase efficiency. The samples were denatured at 94 C for 5 min. Amplification was carried out using 35 standard PCR cycles with a 60 C annealing temperature using a thermal cycler. Aliquots of 15 μl of each PCR sample were subjected to electrophoresis on 1.8% (wt/vol) agarose gels. Bands were visualized, after staining with 0.5 mg/ml ethidium bromide, under UV illumination (Multimage Light Cabinet; Alpha Innotech Corp., San Leandro, CA). The RT-PCR products were identified by sequencing using the BigDye system (Applied Biosystems, Foster City, CA) and an ABI 310 sequencer (PerkinElmer, Wellesley, MA).

Results

Validation of Insl3 antibody

Using the rabbit antirat Insl3 antibody described above, intense immunostaining was detected in the cytoplasm of LCs in control rats at E17.5 but was dramatically reduced in the LCs of all E17.5 animals exposed to DBP *in utero* (Fig. 1), confirming earlier findings based on Insl3 mRNA expression (23). Strong immunostaining for Insl3 was also seen in the LCs of adult rats but was completely absent in the testes of animals in which the LC population had been ablated by treatment with EDS (Fig. 1). No immunostaining was evident in sections from control rat testes when preimmune serum was used in place of the Insl3 antibody (Fig. 1). In controls, Insl3 immunostaining was not uniform throughout the cytoplasm but was often strongest in a circular-ovoid region close to the nucleus, suggesting that the antibody may detect an unprocessed form of Insl3 concentrated within the Golgi apparatus. This interpretation was supported by Western blotting of protein extracted from testes of E17.5 control and DBP-exposed rats and adult control and EDS-treated rats. In samples from control animals (Fig. 2), the antibody detected a protein band corresponding approximately to the molecular weight of unprocessed pro-Insl3 (35), and this band was absent/reduced in samples from DBP-exposed and EDS-treated animals (Fig. 2) and was also absent when the membrane was incubated with preimmune serum, thus confirming the specificity of the antibody.

Developmental expression of Insl3

Immunoexpression of Insl3 was examined at E15.5, E17.5, E19.5, and E21.5 and 4, 10, 15, 25, and 35 d after birth and in adult animals. Results are shown in Fig. 3. Immunopositive LCs were evident in the testes of rats at E15.5, although at this

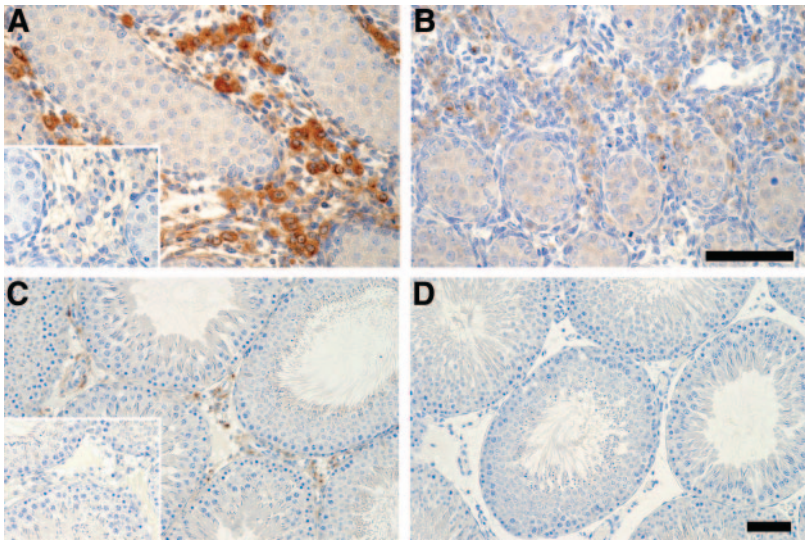

FIG. 1. Representative photomicrographs showing immunoexpression of Insl3 in Leydig cells of testes from fetal and adult rats. Strong immunostaining was detected in testes from control E17.5 (A) and adult (C) animals, but this was reduced markedly in E17.5 rats exposed *in utero* to DBP (B) and was absent in adults in which Leydig cells had been ablated by treatment with EDS (D). *Insets*, Tissue sections incubated with preimmune serum. *Scale bar*, 100 μm.

stage they were relatively sporadic. Immunoexpression of Insl3 was very intense in all LCs at E17.5 and E19.5 but declined sharply at E21.5 and then remained low until 15 d after birth. Immunoexpression of Insl3 increased at 25 d of age and again at 35 d, when intensity of immunostaining was similar to that seen in adult animals (Fig. 3).

Relationship of altered Insl3 expression to altered steroidogenesis/androgen action in fetal life

Because maternal DBP treatment of the rat results in suppression of testosterone production (24, 25, 36) and associated suppression of steroidogenic enzymes such as P450scc (37, 38), we compared the immunoexpression of Insl3 and P450scc in testes of fetuses at E19.5 from mothers treated during pregnancy with vehicle (control), 500 mg/kg·d DBP or 50 mg/kg·d flutamide. The DBP treatment, as expected, led to marked suppression of P450scc immunoexpression in testes of nine of nine animals (from three different litters) and marked suppression of Insl3 in six of nine animals (Fig. 4). In contrast, flutamide exposure had no detectable effect on immunoexpression of either protein in any animal (seven of seven) (Fig. 4), despite causing profound antiandrogenic effects elsewhere in the developing reproductive tract/genitalia (data not shown). This suggests that androgens are not involved in regulation of Insl3 immunoexpression in fetal LCs.

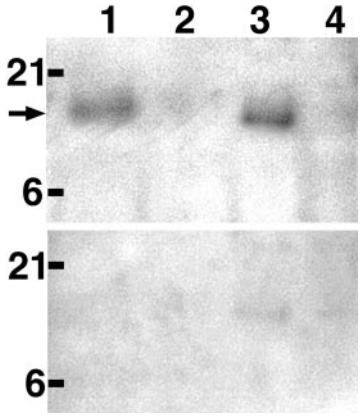

FIG. 2. *Upper panel*, Western blot of proteins extracted from the testes of E17.5 rats exposed *in utero* to vehicle control (lane 1) or DBP (lane 2) and adult control (lane 3) or EDS-treated rats (lane 4), probed with antirat Insl3 antibody. *Lower panel*, Identical samples probed with preimmune serum. Molecular weights are indicated on the *left*. The Insl3 antibody detected a protein (*arrow*) the size of which corresponds approximately to the molecular weight of unprocessed pro-Insl3.

Relationship between Insl3 immunoexpression and testis position in fetal life

DBP exposure *in utero* is clearly associated with reduced expression of both Insl3 mRNA (23) and protein (present study) in the testis and causes abnormalities of gubernacular development (23) and testicular descent (24, 39, 40). We therefore investigated whether fetal testes that were maldescended also exhibited reduced immunoexpression of Insl3 when compared with testes from control and DBP-exposed animals in which a normal testis position was recorded. We restricted these studies to E19.5 and E20.5 because descent of the testes to an inguinal position is normally complete by this age, and it was already established that most, but not all, DBP-exposed animals exhibited suppression of Insl3 expression at both E19.5 (Fig. 4) and E20.5 (not shown). We selected fetal testes from five DBP-exposed animals (from three different litters) in which an abnormally high testis position (adjacent to the kidney) had been recorded at death and compared these with six DBP-exposed animals (from three different litters) in which a normal (inguinal) position of the testes had been recorded; this included some animals from the same litters as those with a high testis position. Additionally, six control animals (from two different litters) with normally descended (inguinal) testes were also used for reference. Results are shown in Table 1. All control animals showed intense, and relatively constant, immunoexpression of Insl3 in all cases. DBP-exposed animals with abnormally descended testes showed reduced immunoexpression of Insl3 in three of five cases, whereas DBP-exposed animals with normally descended testes exhibited reduced immunoexpression of Insl3 in five of six cases. There was thus no consistent relationship between the recorded testis position and Insl3 immunoexpression in the same testis.

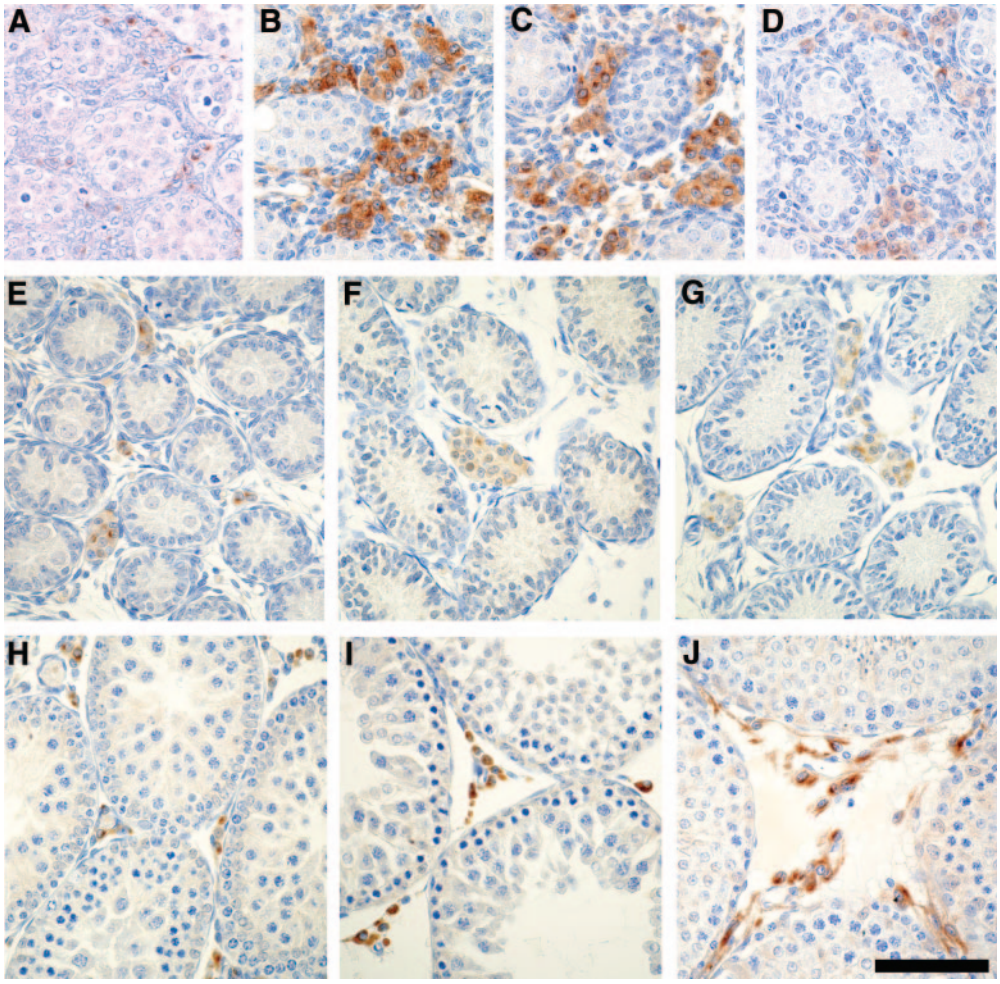

FIG. 3. Immunoexpression of Insl3 throughout development. Panels A–K show Leydig cell immunostaining in testes from E15.5, E17.5, E19.5, and E21.5 and 4, 10, 15, 25, and 35 d and adult rats, respectively. Immunoexpression was very high between E17.5 and E19.5, declined dramatically in late gestation, increased around puberty, and again reached high adult levels by 35 d of age. *Scale bar*, 100 μm.

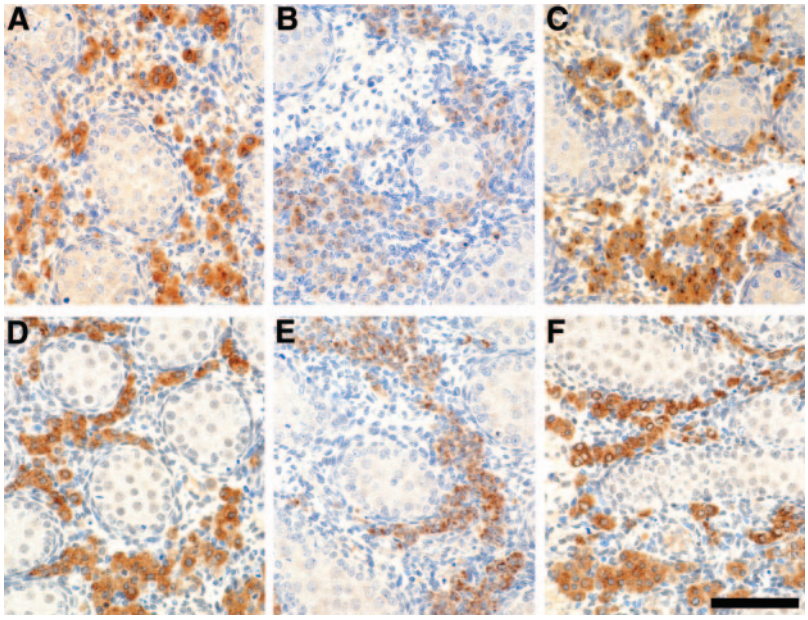

FIG. 4. Comparison of Insl3 (*upper panels*) and P450scc (*lower panels*) immunoexpression at E19.5 after *in utero* exposure to vehicle control (A and D), DBP (B and E), or flutamide (C and F). The lack of effect on either protein of exposure to flutamide is interpreted as evidence for absence of androgen regulation of Insl3 expression in fetal LCs. *Scale bar*, 100 μm.

TABLE 1. Insl3 immunoexpression and testis position in fetal life

	Control	DBP-exposed high testis	DBP-exposed inguinal testis
n	6	5	6
Reduced Insl3 immunoexpression	0	3	5

Table shows numbers of control and DBP-exposed animals at E19.5 and E20.5 in which the testis position was either high (adjacent to kidney) or normal (inguinal) and in which Insl3 immunoexpression was reduced. Testes from all control animals were inguinal.

Immunoexpression of Insl3 and its receptor (LGR8) in the gubernaculum

Because there was no consistent relationship between abnormal position of the testis in DBP-exposed rats and the suppression of Insl3 in these animals, we were prompted to investigate whether Insl3 action on the gubernaculum might occur independently of its testicular expression. We therefore looked for expression of Insl3 in the gubernaculum itself and detected strong Insl3 immunostaining in this tissue at E19.5, which was located in the outer muscular layers (Fig. 5). In this same layer, immunoexpression of the Insl3 receptor (LGR8) was also detected (Fig. 5). However, when we attempted to confirm the expression of Insl3 mRNA in the gubernaculum of fetal rats using RT-PCR, we were unable to detect the presence of any transcript (Fig. 6). It is therefore concluded that the Insl3 immunostaining detected in the gubernaculum is Insl3 protein bound to its receptor.

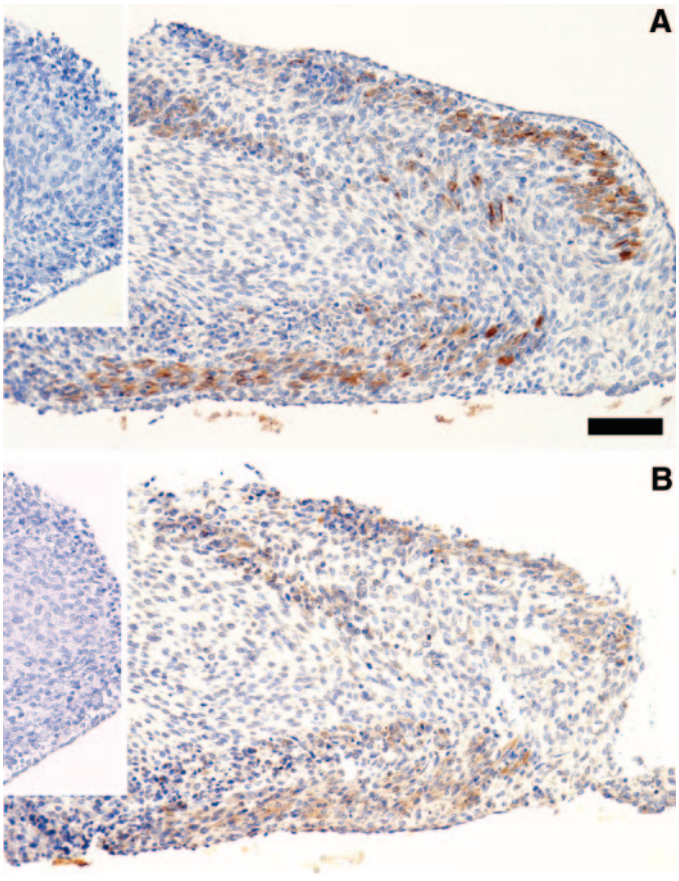

FIG. 5. Immunoexpression of Insl3 (A) and its receptor LGR8 (B) in rat gubernaculum at E20.5. Immunostaining of both proteins was detected in the same region and pattern. Because Insl3 mRNA could not be detected in this organ (see Fig. 6), it is likely that we detected receptor-bound Insl3. *Scale bar*, 100 μm.

Effect of DBP on Insl3 expression in adult rats and relationship with testis position

We examined both testes from eight adult rats (from three litters) exposed *in utero* to DBP, exposure that had resulted in either unilateral or bilateral cryptorchidism in all animals. Cryptorchid testes were characterized by a predominance of Sertoli cell-only seminiferous tubules, whereas scrotal testes were largely normal (not shown; see Ref. 24). Insl3 immunoexpression in LCs of control adult rats was intense and exhibited little variation in intensity between animals (Fig. 7). However, in contrast to what was found in DBP-exposed animals in fetal life, in adulthood Insl3 immunoexpression in 80% (eight of 10) of cryptorchid testes from DBP-exposed animals was markedly and consistently reduced (Table 2 and Fig. 7). Unexpectedly, Insl3 immunoexpression was also consistently reduced in 50% (three of six) of scrotal testes from animals exposed *in utero* to DBP (Table 2 and Fig. 7).

Discussion

In this study we developed, validated, and applied a novel antibody against rat Insl3. This antibody has been used to study expression of Insl3 at the protein level in the LCs of rats throughout fetal and postnatal life and investigate the effect on its expression of *in utero* exposure to DBP or flutamide. In the fetal rat, only sporadic immunoexpression of Insl3 was detectable before E17.5, but intense expression occurred between E17.5 and E19.5 with much lower expression at E21.5. The pattern of Insl3 immunostaining within the LC cytoplasm and our Western blotting results suggest that the antibody detects unprocessed pro-Insl3 in the Golgi apparatus. In postnatal rat LCs, Insl3 expression remained very low until around puberty, at which time its expression steadily increased to reach a maximum level by 35 d of age and into adulthood. We also related variation in LC expression of Insl3 in the fetal testes of rats exposed *in utero* to DBP to their position in an attempt to relate reduced immunoexpression

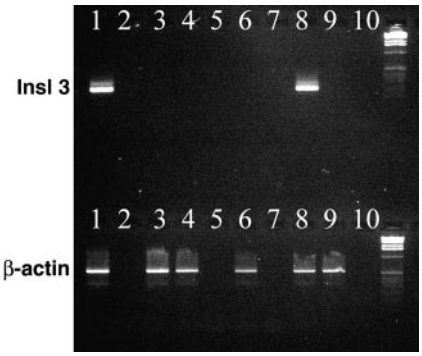

FIG. 6. RT-PCR analysis of Insl3 mRNA in the fetal, postnatal, and adult rat. No Insl3 mRNA was detected in fetal gubernaculum. Lane 1, Adult testis; lane 2, adult testis reverse transcriptase (RT) control; lane 3, E19.5 gubernaculum; lane 4, 4 dpp (days postpartum) gubernaculum; lane 5, 4 dpp gubernaculum RT control; lane 6, adult efferent ducts; lane 7, adult efferent ducts RT control; lane 8, adult Leydig cells; lane 9, adult kidney; and lane 10, PCR-negative control.

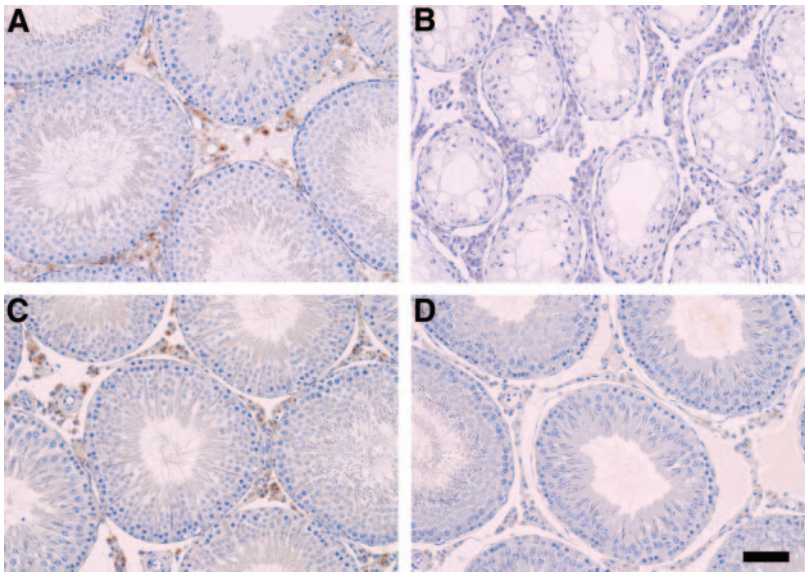

FIG. 7. Immunoexpression of Insl3 in Leydig cells of adult rats after *in utero* exposure to vehicle (controls) or DBP and its relationship to testis position. Expression of Insl3 was high in all control animals (A) but was dramatically reduced in 80% of cryptorchid testes from DBP-exposed animals (B). Expression was unaffected in half of scrotally located testes from DBP-exposed animals (C) but was reduced in the remainder (D). *Scale bar*, 100 μm.

of Insl3 in LCs with failure of normal testis descent; however, we could not find a robust relationship between these two parameters. In contrast, Insl3 immunoexpression was markedly suppressed in the LCs of 80% of cryptorchid testes from adult rats exposed *in utero* to DBP. We also demonstrate that Insl3 protein, but not mRNA, is detectable by immunostaining in the gubernaculum of the fetal rat testis and localizes to the same region as does the Insl3 receptor (LGR8).

The sporadic expression of Insl3 at E15.5 is not unexpected because this time marks the start of fetal LC differentiation (41), whereas the high level of fetal expression from E17.5 to E19.5 is consistent with the role of Insl3 in determining gubernaculum development during the transabdominal phase of testicular descent, which occurs during this period (12). In addition, the developmental pattern of Insl3 protein expression, which we have observed presently, agrees well with previous studies showing that levels of both Insl3 mRNA in mice (20, 42) and serum Insl3 in rats (43), increase coincident with the onset of puberty and with a study that found similar levels of mRNA in fetal and adult rats (9).

One of the main objectives of the present study was to confirm a key role for suppression of Insl3 in the etiology of cryptorchidism in rats exposed *in utero* to DBP (24, 39, 40). Previous studies have shown that Insl3 mRNA expression in the fetal rat testis was suppressed markedly at E18 and E19 after exposure to DBP (23, 44), and our present findings confirm that this is also true at the protein level because immunoexpression was dramatically reduced at both E17.5 and E19.5 when Insl3 expression in control animals was maximal; this is the period during which transabdominal descent of the testis takes place. Our study therefore provides further indirect confirmation of the role of Insl3 suppression in DBP-induced cryptorchidism. However, within individual animals we did not find any consistent correlation between the suppression of Insl3 immunoexpression and the occurrence of abnormally located testes at E19.5. This disparity could indicate that there is not a straightforward relationship between Insl3 protein expression within LC and the levels of this protein in blood, which is what will determine its effect on the gubernaculum. Because DBP exposure also results in marked suppression of fetal testosterone production (23–25, 36, 44, 45) and because androgens also play early and late roles in testicular descent (11), our present findings may indicate that it is the degree of coincident suppression of testosterone and Insl3 in individual animals that determines whether cryptorchidism occurs. In this regard, it is notable that *in vitro* studies have shown that Insl3 and androgens interact to regulate gubernacular development (9, 46). Furthermore, it has been shown that DBP can suppress fetal gene expression of enzymes involved in androgen biosynthesis at lower doses than used in the present study, whereas suppression of Insl3 gene expression occurs only at a dose of 500 mg/kg·d (44). Determining whether DBP-induced suppression of fetal Insl3 protein expression is also dose dependent may help to clarify the respective roles of Insl3 and androgen in testicular descent.

TABLE 2. Insl3 immunoexpression and testis position in adult life

	Control	DBP-exposed cryptorchid testes	DBP-exposed scrotal testes
n	4	10	6
Reduced Insl3 immunoexpression	0	8	3

Table shows numbers of adult animals exposed *in utero* to vehicle control or 500 mg/kg·d DBP in which the testis position was either cryptorchid or scrotal and in which Insl3 immunoexpression was reduced. Testes from all control animals were scrotal.

We were also prompted to explore whether suppression of testosterone production by DBP exposure might itself be a factor leading to suppression of Insl3 in the LCs. We tested this by comparing the effects of *in utero* exposure to DBP or flutamide on the immunoexpression of Insl3 and P450scc because suppression of the latter has been shown to coincide with suppression of fetal testicular testosterone levels (25, 37, 38, 47). This showed clearly that flutamide treatment had no effect on either P450scc or Insl3 immunoexpression, whereas DBP exposure drastically suppressed immunoexpression of both proteins at E19.5. Because other antiandrogenic effects

of flutamide treatment are evident within the testis of these animals (our unpublished data), we consider these data as strong evidence for the absence of androgen involvement in the regulation of Insl3 expression in the fetal LCs.

Our failure to demonstrate a consistent relationship between suppression of Insl3 immunoexpression in fetal LCs and testicular maldescent in individual animals prompted us to consider the possibility that the Insl3 acting on the gubernaculum might not derive from the testis. In this regard, we detected Insl3 immunostaining in the fetal rat gubernaculum at E17.5-E19.5, but RT-PCR analysis failed to confirm the presence of mRNA for Insl3 in gubernacular extracts. However, LGR8 transcripts are known to be expressed in the gubernaculum (9, 48), and in the present study, LGR8 and Insl3 immunostaining were found in the same area of the gubernaculum and in a similar pattern, suggesting that the Insl3 immunostaining which we detected was receptor-bound Insl3.

In common with other studies in rodents and humans (see references above), we found that expression of Insl3 in the adult generation of LCs increases around the time of puberty and that expression in LCs in adult rats was similar to the high level seen in fetal LCs. Together, these findings suggest strongly that, apart from its role in testicular descent, Insl3 may have an alternative role in germ cell support/spermatogenesis. This interpretation is supported by reports that transcripts of the Insl3 receptor LGR8 are present in the testes of adult rats (48, 49) and humans (21) and that, in the rat, transcripts first appear in late puberty (4 wk) and are expressed in the germ cells, but not LCs, of adult animals in a stage-specific manner (49).

In the present study, 100% of adult rats exposed *in utero* to DBP exhibited either unilateral or bilateral cryptorchidism, and many of the seminiferous tubules in cryptorchid testes exhibited a Sertoli cell-only morphology. In contrast to what we found during fetal life, there was a strong correlation between the occurrence of cryptorchidism and suppression of Insl3 expression in adulthood. Because the DBP exposure was confined to fetal life, an obvious possibility is that suppression of Insl3 in the LCs of adult cryptorchid testes is a secondary consequence of cryptorchidism. However, two pieces of evidence suggest that this interpretation is unlikely. First, Insl3 immunoexpression in half of the scrotally located testes of DBP-exposed adult animals was also reduced, whereas such low expression was never observed in any testes from control animals. Second, studies in mice have shown that induction of experimental cryptorchidism does not significantly alter expression of the Insl3 mRNA in the testis (50). Our findings therefore suggest that disturbance of normal testis development, as a result of *in utero* exposure to DBP, leads both to cryptorchidism and maldevelopment/malfunction of the adult LC population and that one manifestation of the latter is suppression of Insl3 expression.

In conclusion, the present findings demonstrate that significant age-related changes in expression of Insl3 protein expression occur in both the fetal and adult generation of LCs in the rat. Our finding of major suppression of Insl3 immunoexpression at E17.5-E19.5 in the testes of most rats exposed *in utero* to DBP fits with the high incidence of testicular maldescent in such animals, although in individual animals no consistent relationship between testicular malposition and suppression of Insl3 immunoexpression was evident. This finding may indicate that cryptorchidism results from the combined suppression of both Insl3 and testosterone, rather than just because of Insl3 suppression. This conclusion might explain the relative paucity of evidence from human studies for a key role for Insl3 mutations/polymorphisms in cases of cryptorchidism (19) and would fit with current concepts that disorders such as cryptorchidism, which may comprise a testicular dysgenesis syndrome (4, 5), stem from a fundamental dysfunction of testicular LCs and/or Sertoli cells rather than from a single defect. Our finding of increased expression of Insl3 in adult LCs as they mature adds to evidence in the literature to suggest a role(s) for this hormone in the adult testis, and in this regard our findings also show that Insl3 expression in adult cryptorchid testes of rats exposed *in utero* to DBP is grossly suppressed.

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